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Non-O1/Non-O139 *Vibrio cholerae* Avian Isolate from France Cocarrying the bla_{VIM-1} and bla_{VIM-4} Genes

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We describe here a non-O1/non-O139 *Vibrio cholerae* isolate producing both VIM-1 and VIM-4 carbapenemases. It was isolated from a yellow-legged gull in southern France. The bla_{VIM} genes were part of a class 1 integron structure located in an IncA/C plasmid. This study emphasizes the presence of carbapenemase genes in wildlife microbiota.

Multidrug-resistant *Vibrio cholerae* strains have been increasingly reported worldwide (1). However, data on resistance to third-generation cephalosporins, mostly via genes encoding extended-spectrum β-lactamase (ESBL) (2, 3) or cephalosporinase determinants (4) are limited. Carbapenemase-mediated resistance in *Vibrio* spp. has been reported only in India, where clinical and environmental *V. cholerae* isolates carrying the NDM-1 metalloenzyme were described in several studies (4–6). We describe here an avian strain of *V. cholerae* that was isolated in southern France and that coharbors the bla_{VIM-1} and bla_{VIM-4} carbapenemase genes.

In April 2013, 93 cloacal swab samples from juvenile unfledged yellow-legged gulls (*Larus michahellis*) breeding on the island of Carteau, Port-Saint-Louis, France, were screened for bacteria resistant to broad-spectrum β-lactam antibiotics. Briefly, swab samples were inoculated in Trypticase soy broth (Thermo Fisher Scientific) and grown at 37°C for 24 h. Samples were then subcultured in ESBL agar plates (bioMérieux, Marcy l'Etoile, France) and were examined after 24 and 48 h of incubation. *Enterobacteriaceae* resistant to multiple drugs via different resistance mechanisms (e.g., third-generation cephalosporin-resistant *Escherichia coli* and *Proteus mirabilis* harboring plasmid-mediated cephalosporinase genes or ESBL genes) were recovered (7; our unpublished data). In addition, a *V. cholerae* strain showing resistance to third-generation cephalosporins was detected. No other multidrug-resistant *V. cholerae* strain was recovered. Species identification was performed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany). Moreover, PCR analysis of the *rfb* gene cluster (8), the cholera toxin *ctxA* gene (9), and the colonization factor *tcpA* gene (9) revealed a nontoxigenic, non-O1/non-O139 isolate.

Susceptibility testing was performed using the disk diffusion method on Mueller-Hinton agar and was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (version 5.0) (http://www.eucast.org/clinical_breakpoints/) (10). The strain was intermediate or resistant to most β-lactam antibiotics, except aztreonam. The MICs for amoxicillin, cefotaxime, ceftazidime, imipenem, ertapenem, doripenem, and meropenem were determined in the parental strain and the transconjugant with the Etest method (bioMérieux, Marcy l'Etoile, France) (Table 1). Metallo-β-lactamase production was observed by using the carbapenemase/metallo-β-lactamase confirmative identification pack (Rosco Diagnostica NeoSensitabs, Eurobio, Courtaboeuf, France). Specifically, reduced susceptibility to meropenem was corrected by the addition of dipicolinic acid, while the addition of cloxacillin and boronic acid had no effect. ESBL production was excluded with the double-disk synergy test (11), while culture in Mueller-Hinton agar impregnated with 2 ml of 5×10^{-3} M EDTA restored the activity of all β-lactam antibiotics, as previously described (12). Susceptibility testing using the disk diffusion method showed that fluoroquinolones, chloramphenicol, cotrimoxazole, and tetracycline remained active. Only tobramycin showed intermediate susceptibility among the aminoglycosides, while amikacin, isepamicin, netilmicin, and gentamicin remained active.

Detection of the most prevalent carbapenemase genes (including bla_{KPC}, bla_{VIM}, bla_{OXA-48}, and bla_{IMP-1}), assessed by multiplex PCR as previously described (13), and of the bla_{NDM} gene (14), assessed by PCR assay, gave a positive result for the bla_{VIM} gene. This was confirmed by simplex PCR assay using the primers VIM_F (5′-AGTGGTGAGTATCCGACAG-3′) and VIM_R (5′-TGCAACTTCATGTTATGCCG-3′). Bidirectional sequencing performed using the BigDye Terminator v3.1 cycle sequencing kit

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TABLE 1 Susceptibility of parental and recipient strains

Antibiotic	MIC (mg/liter) for:		
	Parental strain (*V. cholerae*)	Recipient strain (J53 *E. coli*)	J53 *E. coli*
Amoxicillin	>256	>256	2
Cefotaxime	4	8	0.064
Ceftazidime	6	24	0.064
Aztreonam	0.38	0.032	0.064
Imipenem	3	4	0.25
Ertapenem	0.19	0.064	0.064
Meropenem	0.5	0.75	0.064
Doripenem	0.75	0.75	0.064
Amikacin	2	0.5	0.5
Gentamicin	1.5	0.25	0.25
Tobramycin	2	0.75	0.5

TABLE 2 Primers used for PCR mapping of the cassette network

Primer name	Primer sequence (5′ to 3′)
VIM-L1	TCATTGTCCGTGATGGTGATGA
VIM-L2	CCGGGCGGTCTAGACTTGCT
VIM-R1	CGATATGCGACCAAACACCATC
VIM-R2	GCCATTCAGCCAGATCG
attI1	GGCATCCAAGCAGCAAGCGCGTT
sul1	GTCCGACATCCACGACGTCTGATC

(Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3730 XL capillary sequencer identified both bla_{VIM-1} and bla_{VIM-4} genes.

To characterize the genetic environment of the bla_{VIM} genes, the amplicons of parental and recipient strains were analyzed by PCR mapping and sequencing using specific primers (Table 2) (GenBank accession number KR262557). Both bla_{VIM-1} and bla_{VIM-4} genes were part of the same class 1 integron (Fig. 1), located in the IncA/C plasmid. These two carbapenemase gene cassettes flanked an *aac(6′)-IIc* gene cassette that confers resistance to aminoglycosides. A PcS (strong) promoter variant, divergent to the integrase gene, was identified in the class 1 integron, with a functional P2 promoter located downstream of the PcS in the *attI1* site. It resulted from the insertion of three G residues. The PcS-P2 association has rarely been described in class 1 integrons and might confer high-level gene cassette expression (15). A similar integron containing the bla_{VIM-1} and *aac(6′)-IIc* genes was previously described in an *Enterobacter cloacae* clinical isolate from Greece (GenBank accession number AY648125) (16), but this is the first description of a class 1 integron with two bla_{VIM} variants. It may be hypothesized that the presence of two bla_{VIM} genes in a single integron might enable better plasticity in the case of rearrangements of the cassette network under selective pressure caused, for instance, by antibiotics.

Mating experiments were performed on agar plates, as previously described (6), at 25°C and 37°C using the rifampin-resistant *E. coli* J53 strain as the recipient, with a donor-to-recipient ratio of 4:1. Transconjugants were selected on Drigalski agar (Bio-Rad) containing 250 mg/liter rifampin and 4 mg/liter cefotaxime. A transconjugant that coharbored the bla_{VIM-1} and bla_{VIM-4} genes was obtained from the *V. cholerae* isolate at 25°C, with a transfer frequency of 3×10^{-6} transconjugants per recipient. PCR mapping showed that the genetic structure that harbored both bla_{VIM} genes was the same in the parental and recipient strains. No transfer was obtained at 37°C, despite repeated attempts. The plasmid relaxase gene typing (PRaseT) method, which allows detection of the major replicon groups, and a PCR-based replicon typing method revealed the presence of an IncA/C plasmid in the parental and recipient strains (17, 18). Conversely, the SXT integrative and conjugative element, which is a major resistance determinant in *V. cholerae* (1), was detected in only the parental strain by PRaseT. No other typeable mobile genetic element was found. Plasmid content analysis using the method of Kado and Liu (19) revealed only one plasmid of ~150 kb in both the parental and recipient strains (see Fig. S1 in the supplemental material). These results suggest that the bla_{VIM-1} and bla_{VIM-4} genes were carried by the broad-host-range IncA/C transmissible plasmid, a widespread bla_{VIM}-carrying genetic element (20) and a common resistance determinant in *V. cholerae* (21).

Although nonepidemic, non-O1/non-O139 *V. cholerae* isolates are human pathogens that may cause diarrhea and extraintestinal infections (21). Fluid resuscitation remains the first-line therapy, but the use of antibiotics allows decreased symptom duration and pathogen dissemination. This is, to our knowledge, the first description of a non-O1/non-O139 *V. cholerae* isolate harboring bla_{VIM} carbapenemase genes worldwide and the first description of a carbapenemase-producing *V. cholerae* in Western countries.

Here, its identification in an animal microbiota brings new insights into the presence of such strains in wildlife ecosystems. Walsh et al. (6) previously described NDM-1–carrying *V. cholerae* in seepage and tap water samples collected in New Delhi, India, emphasizing the transfer frequency of this metalloenzyme in various recipient species at environmentally relevant temperatures. In their study, conjugative transfer of IncA/C plasmids harboring bla_{NDM-1} in *V. cholerae* did not happen at 37°C, and average transfer frequencies of 10^{-6} and 10^{-4} were observed at 25°C and 30°C, respectively. Similarly, our study found that the plasmid could transfer at 25°C with comparable frequencies, whereas conjugation attempts at 37°C were unsuccessful. This suggests that optimal transfer conditions would mainly occur in the environment rather than in the gut. The presence of metalloenzymes on different genetic locations (IncA/C and nontypeable plasmids [4, 6], chromosome [5, 6]) in *V. cholerae* is of concern because it indicates that they can spread easily on various mobile genetic elements.

Yellow-legged gulls inhabit mostly coastal regions across the Mediterranean, where they breed in dense colonies. As they can fly long distances across the European and northern African borders, especially during their first year of life, they could play a role in the dissemination of bla_{VIM}-harboring *V. cholerae*. Based on the feeding habits of yellow-legged gulls (they mainly rely on anthropogenic resources) and their microbiota diversity (including *E. coli*), it is reasonable to think that they are an important zoonotic reservoir of multidrug-resistant organisms, including bla_{VIM}-carrying bacteria. This is consistent with reports highlighting the increasing prevalence of carbapenemase-producing microorganisms in wildlife microbiota (22). Moreover, their direct exposure to human activities might play a role in the spread of antibiotic resistance, as previously illustrated by ESBL-positive *E. coli* isolates with similar genetic background recovered among gulls and humans in several countries, including southern France (23). Further studies are

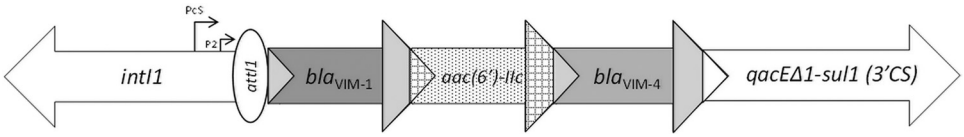

FIG 1 Linear map of the class 1 integron harboring both bla_{VIM-1} and bla_{VIM-4} genes. Arrows, relative gene size and direction of transcription; black arrows, gene cassette promoters PcS and P2.

needed to assess the prevalence of carbapenemase genes and their genetic background in wildlife microbiota.

Nucleotide sequence accession number. The sequence of the class 1 integron harboring both bla_{VIM-1} and bla_{VIM-4} genes has been submitted to GenBank under accession number KR262557.

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